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Cloning of shRNA and sgRNA Templates into Cellecta Expression Vectors

User Manual

V5, 7/16/15

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A. Background

The protocols below provide the instructions on how to phosphorylate shRNA or sgRNA template oligos, ligate them to cloning vectors, transform competent cells, and grow plasmid DNA to generate plasmid expression constructs. For packaging of constructs into lentiviral particles, please refer to the User Manual for Packaging and Transduction of Lentiviral Constructs. Please read the entire user manual before proceeding with your experiment.

B. Required Materials

For Digestion of Uncut Cloning Vector

- BbsI enzyme (Recommended: New England BioLabs, Cat.# R0539L)

For Phosphorylation and Annealing of shRNA or sgRNA Template Oligonucleotides

- T4 Polynucleotide Kinase and 10X Reaction Buffer (Recommended: New England BioLabs T4 Polynucleotide Kinase, 10 U/µl, Cat. # M0201S)
- rATP (Recommended: GE/Amersham, Cat. # 27-2056-01)

For Ligating and Transforming shRNA or sgRNA Constructs

- *Linearized* Cellecta shRNA Expression Vector or CRISPR Expression Vector

- T4 DNA Ligase and 10X Ligation Reaction Buffer (Recommended: New England BioLabs, T4 DNA Ligase, 400 U/μl. Cat. # M0202S): ***Before using, dilute T4 DNA-ligase 10-fold with 1X T4 DNA ligase buffer to 40 U/μl.)***
- Competent *E. coli* cells (RecA-) (Recommended: Invitrogen, OmniMAX™ 2 T1 Phage-resistant cells, Cat. # C8540-03)
- Petri plates containing LB Agar media with 100 μg/ml Ampicillin or Carbenicillin

For Screening shRNA or sgRNA Inserts

- Taq DNA polymerase, and 10X reaction buffer (Recommended: BDB Clontech Titanium™ Taq DNA polymerase, Cat. # 639208)
- dNTP mix (Recommended: GE/Amersham, dNTP set, Cat. # 27-2035-01)
- Thermal Cycler
- 3% 1X TAE Agarose gel
- PCR Screening Primers for your vector*

** Please check the Product Analysis Certificate (PAC) that came with your product.*

For Purifying shRNA and Constructs after Cloning

- Plasmid purification kit (Recommended: QIAGEN Endotoxin-free Plasmid Kit. The following kit combinations can be used for Midi scale preparation of endotoxin-free DNA:
  - QIAfilter Plasmid Midi Kit, Cat. # 12243, and EndoFree Plasmid Maxi Kit, Cat. # 12362
  - QIAfilter Plasmid Midi Kit, Cat. # 12243, and EndoFree Plasmid Buffer Set, Cat. # 19048
- Please visit the QIAGEN website to download the specialized protocol that is not contained in the user manual: http://www1.qiagen.com/literature/protocols/pdf/QP15.pdf

C. Design of shRNA Template Oligos for shRNA Expression Vectors

The general design of shRNA template oligonucleotides is:

5′-ACCGG-(21sense)-(Stem-Loop-Stem)-(21antisense)-TTTT-3′

Sense oligo:
5′-accgGNNNNNNNNNNNNNNNNNNNNNGTTAATATTCATAGCNNNNNNNNNNNNNNNNNNNNTTTT-3′

Antisense oligo:
5′-cgaaAAAANNNNNNNNNNNNNNNNNNNNNGCTATGAATATTAACNNNNNNNNNNNNNNNNNNNNC-3′

D. Design of sgRNA Template Oligos for CRISPR Expression Vectors

The general design of sgRNA template oligonucleotides is:

5′-ACCG-(20nt guide)-(scaffold/tracrRNA)-TTTT-3′

Sense Oligo:
5'accgNNNNNNNNNNNNNNNNNNNgttttagagctagaaatagcaagttaaaataaggctagtccgttatcaact
tgaaaaagtggcaccgagtcggtgctttt-3'

Antisense Oligo:
5'cgaaaaaagcaccgactcggtgccacttttcaagttgataacggactagccttattttaacttgctatttctag
ctctaaaacNNNNNNNNNNNNNNNNNNNN-3'

Guide: NNNNNNNNNNNNNNNNNNNN

Scaffold/tracrRNA:
5'gttttagagctagaaatagcaagttaaaataaggctagtccgttatcaacttgaaaaagtggcaccgagtcggtg
c-3'

E. Cloning of shRNA or sgRNA Template Oligonucleotides into an Expression Vector

1. Phosphorylate and Anneal the shRNA Template Oligonucleotides

Note: This protocol was developed for regular non-phosphorylated oligos. If your oligonucleotides are already phosphorylated, dilute them to 10 μM in 1X T4 polynucleotide kinase buffer, heat at 95°C for 2 min and anneal as in steps 1.d-1.e below.

a. Dissolve the shRNA or sgRNA template oligonucleotides in an appropriate amount of deionized water to a final concentration of 20 μM.

b. Set up 20 μl phosphorylation/annealing reactions for each shRNA or sgRNA template:

1 μl	Sense Strand shRNA or sgRNA template oligo (20 μM) *
1 μl	Antisense Strand shRNA or sgRNA template oligo (20 μM) *
2 μl	10X T4 Polynucleotide Kinase Buffer
2 μl	5 mM ATP
13 μl	Deionized water
1 μl	T4 Polynucleotide Kinase (10 U/μl)
20 μl	Total volume

** For the insert-minus control, use 2 μl deionized water in place of the sense and antisense strands.*

c. Incubate the phosphorylation reaction at 37°C for 30 minutes in a thermal cycler.

d. Heat the reaction mix to 95°C for 2 min in a thermal cycler.

e. Turn off the thermal cycler and let it cool to room temperature.

f. Take a 2 μl aliquot from the reaction (1 μM shRNA or sgRNA template), and make a 1:5 dilution by adding 8 μl of 1X T4 Kinase buffer. Mix well.

g. Use 0.5 μl of the diluted shRNA or sgRNA template (0.2 μM) for the following ligation reaction.

2. Ligate the shRNA or sgRNA Template into Linearized shRNA or sgRNA Lentivector

a. Set up 10 μl ligation reactions for each phosphorylated shRNA or sgRNA template as follows:

1.0 μl	Linearized shRNA or sgRNA Expression Vector (10 ng/μl) *(see Required Materials)*
0.5 μl	Phosphorylated ds shRNA or sgRNA template (step 1; 0.2 μM) *
1.0 μl	10X T4 DNA Ligase Buffer
6.5 μl	Deionized water
1.0 μl	T4 DNA ligase (40 U/μl) **
10.0 μl	Total volume

** For negative control use insert-minus.*

*** Dilute T4 DNA ligase (400 U/μl) 10-fold to 40 U/μl with 1X T4 DNA ligase buffer if you are using New England BioLabs enzyme.*

b. Incubate the ligation reaction at 16°C for 1-2 hrs.

3. Transform *E. coli* with the ligation product

a. For each shRNA or sgRNA template, use the whole volume of ligation product for transformation.

b. Follow the manufacturer's protocol for transforming the competent cells.

c. Plate an appropriate amount of cells on LB plates with 100 μg/ml ampicillin or carbenicillin and grow overnight at 37°C.

d. You should expect to get at least 10-fold more colonies in experimental samples in comparison with negative control (vector-only ligation reaction).

F. Identify clones with the target shRNA or sgRNA template

1. Prepare colony cultures

a. Randomly pick up 10 well-separated colonies from each plate, and grow each clone in 100 μl of LB Broth with 100 μg/ml ampicillin or carbenicillin at 37°C for 2 hours with shaking.

b. Take 1 μl of each bacteria culture for PCR screening and continue to grow the culture for another 6 hours.

c. Store the bacterial culture at 4°C.

2. Screen for shRNA or sgRNA template inserts

a. Prepare a PCR master mix for each clone you would like to screen for the presence of an shRNA or sgRNA template insert as follows:

1 rxn	10 rxn	Composition
0.5 μl	5 μl	Forward PCR Primer (10 μM)*
0.5 μl	5 μl	Reverse PCR Primer (10 μM)*
0.5 μl	5 μl	50X dNTP mix (10 mM of each)
2.5 μl	25 μl	10X PCR Reaction Buffer
19.5 μl	195 μl	Deionized water
0.5 μl	5 μl	Taq DNA Polymerase (5 U/μl)
24.0 μl	240 μl	Total volume

**See the Product Analysis Certificate for your plasmid to find the required primer sequences.*

b. Mix the master mix very well and aliquot 24 μl into each well of a 96-well PCR plate or individual tubes.

c. Add 1 μl of each bacterial culture from B.1 into each well or tube from B.2.b. Mix.

d. Proceed with PCR using the following program:

94°C, 4 min	1 cycle
94°C, 0.5 min, then 68°C, 1 min	25 cycles
68°C, 2 min	1 cycle

e. Take 5 μl of PCR product from Step d and run it on a 3% agarose/EtBr gel in 1X TAE buffer.

f. Confirm identity of shRNA or sgRNA template inserts by sequence analysis of positive PCR products using the Forward PCR primer.

G. Purify Plasmid shRNA or sgRNA Construct

1. Take 15 μl of each positive bacteria culture, inoculate each clone in 10 ml of LB broth media with 100 μg/ml ampicillin or carbenicillin, and grow overnight at 37°C with shaking.
2. Purify shRNA or sgRNA lentivector construct plasmid DNA in Midi or Maxi scale using an Endotoxin-free plasmid purification kit.

H. Technical Support

For additional information or technical assistance, contact us by phone or email:

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For the latest technical news and updates, visit Cellecta's blog at:

http://www.cellecta.com/company/blog-news/

I. Safety Guidelines

The HIV-based lentivector system is designed to maximize its biosafety features, which include:

- A deletion in the enhancer of the U3 region of 3'ΔLTR ensures self-inactivation of the lentiviral construct after transduction and integration into genomic DNA of the target cells.
- The RSV promoter upstream of 5'LTR in the lentivector allows efficient Tat-independent production of lentiviral RNA, reducing the number of genes from HIV-1 that are used in this system.
- Number of lentiviral genes necessary for packaging, replication and transduction is reduced to three (gag, pol, rev). The corresponding proteins are expressed from different plasmids lacking packaging signals and share no significant homology to any of the expression lentivectors, pVSV-G expression vector, or any other vector to prevent generation of recombinant replication-competent virus.
- None of the HIV-1 genes (gag, pol, rev) are present in the packaged lentiviral genome, as they are expressed from packaging plasmids lacking packaging signal—therefore, the lentiviral particles generated are replication-incompetent.
- Lentiviral particles will carry only a copy of your expression construct.

Despite the above safety features, use of HIV-based vectors falls within NIH Biosafety Level 2 criteria due to the potential biohazard risk of possible recombination with endogenous lentiviral sequences to form self-replicating virus or the possibility of insertional mutagenesis. For a description of laboratory biosafety level criteria, consult the Centers for Disease Control Office of Health and Safety Web site at:

http://www.cdc.gov/biosafety/publications/bmbl5/bmbl5_sect_iv.pdf

It is also important to check with the health and safety guidelines at your institution regarding the use of lentiviruses and follow standard microbiological practices, which include:

- Wear gloves and lab coat at all times when conducting the procedure.
- Always work with lentiviral particles in a Class II laminar flow hood.
- All procedures are performed carefully to minimize the creation of splashes or aerosols.
- Work surfaces are decontaminated at least once a day and after any spill of viable material.
- All cultures, stocks, and other regulated wastes are decontaminated before disposal by an approved decontamination method such as autoclaving. Materials to be decontaminated outside of the immediate laboratory area are to be placed in a durable, leakproof, properly marked (biohazard, infectious waste) container and sealed for transportation from the laboratory.

J. Terms and Conditions

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Cellecta grants the end user (the "Recipient") of the CRISPR Pooled Lentiviral sgRNA Libraries and Vector (the "Product") a non-transferable, non-exclusive license to use the reagents for internal research use only as described in the enclosed protocols; in particular, research use only excludes and without limitation, resale, repackaging, or use for the making or selling of any commercial product or service without the written approval of Cellecta, Inc. - separate licenses are available for non-research use or applications. The Product is not to be used for human diagnostics or included/used in any drug intended for human use. Care and attention should be exercised in handling the Product by following appropriate research laboratory practices.

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